Characterization of three novel monoclonal anti-Ok^a

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Anti-Ok^a was first described by Morel and Hamilton in 1979. The Ok^a antigen has a very high incidence, and only eight probands that are Ok(a–) have been found; all are of Japanese heritage. In this study, we describe the generation and characterization of three novel monoclonal antibodies (Mabs), MIMA-25, MIMA-144, and MIMA-149. The reactivity of these three Mabs was compared with the original human polyclonal anti-Ok^a. Mice were immunized with transfected HEK cells to induce an immune response, and the spleen B lymphocytes were fused with mouse myeloma X63-Ag8.653 cells to form antibody-secreting hybridomas. The resulting Mabs were tested serologically, by flow cytometry, and by immunoblotting. The specificity of each antibody was determined after excluding specificities to common antigens in the Rh, Kell, Duffy, Kidd, MNS, Lewis, Lutheran, P1, Colton, Diego, Xg^a, and Dombrock blood group systems. In each case only the Ok(a–) RBC sample was nonreactive. The Mabs and the original human anti-Ok^a each have a unique pattern of reactivity when tested with enzyme-treated cells; however, none were reactive by immunoblotting. We have generated three novel anti-Ok^a Mabs: MIMA-144 is an indirectly agglutinating IgG2b antibody, and MIMA-25 and MIMA-149 are directly agglutinating antibodies (IgM and IgA, respectively), underscoring their usefulness as typing reagents for the clinical laboratory.
***Immunohematology* 2009;25:174–178.**

Key Words: anti-Ok^a, CD147, blood groups, hybridoma, monoclonal antibodies, transfection, flow cytometry

Commercial reagents for antigen typing RBCs have commonly been derived from polyclonal human serum collected from hyperimmunized donors. However, the direct immunization of human subjects is no longer recommended by the FDA, thus the supply of human polyclonal reagents is rapidly diminishing and becoming increasingly more expensive to maintain. Kohler and Milstein first reported the successful development of a monoclonal antibody–producing hybridoma cell line.[1] Monoclonal antibodies (Mabs) are now commonly used as blood typing reagents, replacing the need for human polyclonal reagents.[2]

Although producing antibodies to carbohydrate antigens (ABO, Lewis) was relatively easy, developing Mabs to other types of antigens has proven to be much more difficult.[3–5] Furthermore, the early methods applied only to the murine model. The production of Mabs for human antigens requires a highly immunized donor, the viral transformation of human B lymphocytes, and a successful fusion with a mouse myeloma cell line.[6] Transformation was needed to expand the number of human B lymphocytes for fusion, and, fortunately, it was found to improve the susceptibility of B lymphocytes for somatic cell hybridization by up to 25-fold.[7-8] Unfortunately, heterohybridoma cell lines have been found to be highly unstable, and thus more likely to stop secreting antibody after 6 to 12 months.[9-10] As our work in the production of Mabs to blood group antigens developed, so too did the use of novel approaches to achieve an immune response in the mouse that would lead to the development of murine-based Mabs to human blood group antigens.[11–13]

The OK Blood Group System

The antibody Ok^a was named for the family name of the proband (S.Ko.G) whose plasma reacted with all normal RBCs.[14] Born of consanguineous parents on a small island off the coast of Onomichi, Honshu, Japan, the proband also had two siblings who were Ok(a–). Anti-Ok^a reacted by the antiglobulin test with blood samples from more than 4000 Japanese donors. Since its discovery there have been only eight Ok(a–) probands identified, all in persons of Japanese heritage.[15] Of more than 12,000 blood donors of various ethnic backgrounds tested in the United States, no other Ok(a–) individuals have been found.

The Ok^a antigen is the only antigen in the OK blood group system and was assigned the ISBT # 024001 after it was located on CD147 glycoprotein and thereby was eligible for blood group system status. The OK glycoprotein is a single-pass type I membrane glycoprotein with two Ig superfamily (IgSF) domains and a short transmembrane and cytoplasmic domain. Sequence analysis showed the N-terminal 30 amino acids are identical to the predicted sequence of the M6 leukocyte activation antigen, which is a member of the IgSF. The *OK* gene consists of seven exons distributed over 1.8 kbp of gDNA, and it was localized using somatic cell genetics to chromosome 19, subregion 19p13.2-pter.[16] The rare Ok(a–) phenotype is the result of an amino acid substitution in the Ok glycoprotein (Ok(a+) > Ok(a–); E92K) encoded by exon 4 of *OK*.[17]

The Ok glycoprotein (CD147) is the most common protein found in the membranes of leukocytes having a role in cell-cell interaction and in signal transduction, and it is thought to act as a tumor growth factor stimulating the production of collegenase and other cell invasion inducers. This glycoprotein is almost ubiquitous in that it is found on blood cells (RBCs, WBCs, and platelets) and in tissues of the renal cortex, liver, pancreas, colon, cervix, testes, smooth muscle, and brain.

The first monoclonal anti-Ok^a was produced by Andrews et al. in 1983.[18] in this paper we will describe the production and characterization of three new murine antibodies to the high-incidence Ok^a antigen. They were evaluated serologically for specificity, titer and score, immunoglobulin isotype, and reactivity pattern when tested with enzyme-treated RBCs.

They were also tested by flow cytometry and by immunoblotting.

Materials and Methods

Immunization Protocol

Two mice were immunized biweekly for 6 weeks with 2 million human embryonic kidney (HEK) cells that had been transfected with human cDNA along with a CpG adjuvant specific for murine Mab production.[18] After the immune response was verified by serum hemagglutination, the spleen was removed and splenocytes obtained by pulverizing the spleen with sterile glass slides.

Cellular Fusions

Fusions between the splenocytes and the mouse myeloma cell line (X63-Ag8.653) were done using polyethylene glycol (PEG, mol. wt., 3450) in a ratio of 3:1 spleen to myeloma cells, respectively. Fusions were performed by drop-wise addition of 1.0 mL of PEG to the washed, dry cells in a conical centrifuge tube with constant mixing for 60 seconds. This was followed immediately by submerging the centrifuge tube into a 37°C water bath for 90 seconds with constant mixing, after which a drop-wise addition of 1 mL of sterile phosphate buffered saline (PBS, pH 7.3, Gibco, Grand Island, NY) without calcium and magnesium was added for 60 seconds. This step was repeated, and then followed by the addition of 2 mL of PBS for 120 seconds. This was continued until the total volume was approximately 15 mL. The now fused sample was rested for 15 minutes in the 37°C CO_2 incubator, and then gently centrifuged. The PBS was removed and the cells were suspended in culture media (HAMS/DF-12; Sigma Chemicals, St. Louis, MO) that had been supplemented with 10 percent fetal calf serum and hypoxanthine-adenine-thymidine (HAT) media (Sigma). The fused cells were gently mixed, then spread over 96-well flat-bottom culture trays at 100 µL per well. After 1 week, fresh culture media supplemented with hypoxanthine-thymidine and gentamicin, but without aminopterin (StemCell Technologies, Vancouver, BC), was added as a selection media. After another week, the resulting hybrids were screened by hemagglutination in v-well microtiter trays for antibody production.

Hemagglutination Assays of 96-Well V-Well Trays

The supernatant culture fluid was assayed for reactivity with selected RBCs by 96-well v-bottom microtiter trays (Greiner Labs, Germany, www.GBO.com). For v-well assays, 50 µL of supernatant fluid (SNF) was removed from each well of the fusion trays and placed in the corresponding well on the v-well assay tray. To this, 25 µL of a 1% suspension of human Ok(a+) RBCs in PBS was added, followed by mixing on a mechanical plate mixer (Labline Instruments Inc., Melrose Park, IL). The reactions were allowed to settle for 30 minutes and then observed for direct agglutination. The cells were washed in PBS, and 50 µL of anti-mouse IgG diluted 1:100 in 6 percent BSA and PBS was then added to each well. The trays were again mixed, allowed to settle, and observed for indirect agglutination.

Hemagglutination in Tube and Gel Cards

Standard hemagglutination assays were done in test tubes using one drop of a 3 percent RBC suspension in PBS plus two drops of Mab. MIMA-25 was incubated at room temperature for 30 minutes with Lo-Ion (Immucor/Gamma, Norcross, GA) added to enhance reactions, then spun and read. MIMA-149 was incubated at room temperature for 30 minutes, spun, and read. MIMA-144 was incubated for 30 minutes at room temperature and tested by IAT using anti-mouse IgG (The Binding Site, San Diego, CA) that had been diluted 1:50 in 6% bovine serum albumin (BSA) in PBS.

For the IgG gel card (DiaMed ID-PNH, Cressier, Switzerland), 50 µL of a 1 percent suspension of Ok[a](+) RBCs and 25 µL of the Mab were added to each column, incubated at 37°C for 15 minutes, and then centrifuged and read for agglutination.

Cloning by Limiting Dilution

When a colony of hybrid cells is found to be producing the antibody of interest, the hybrids are counted and diluted such that there will be a finite number of cells in a 20-mL dilution. This dilution is done so that there will statistically be a limited number of cells per well when they are pipetted into 96-well flat-bottom cloning trays. Three clonings were done to ensure that the final product is from a monoclonal cell line. The initial cloning was done at 10, 5, and 2 cells per well to promote the development of a stable hybridoma cell line. The next two clonings were done at a dilution of 5, 2, and 1 cell per well. The final antibody was selected from the last set of cloning trays.

Enzyme Treatment of RBCs

Human RBCs that had been pretreated with a variety of enzymes or reducing chemicals were tested by standard hemagglutination methods to determine whether the Ok[a] antigen detected by the antibodies was resistant or sensitive to the enzyme effect on the protein.[19] This included treatment with papain, trypsin, α_1-chymotrypsin, pronase, neuraminidase, and 0.2 M dithiothrietol (DTT). The original human anti-Ok[a] was tested in parallel.

Flow Cytometric Analysis

A 1% RBC suspension of test RBCs was first incubated with 500 µL of each Mab and incubated at 37°C for 30 minutes, and then washed with PBS. The antibody-sensitized RBCs were then stained with fluoresceinisothiocyanate (FITC)-tagged anti-mouse antiglobulin (IgG from Vector Labs, Burlingame, CA; IgA and IgM from Southern Biotechnology Associates, Birmingham, AL) by incubating in the dark at 4°C for a minimum of 15 minutes with frequent mixing to ensure adequate staining. The analytes were brought up to a final volume of 250 µL with PBS. Data were

acquired on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) and analyzed with Cell Quest Pro software (BD Biosciences).

Results

MIMA-25, MIMA-144, and MIMA-149 are specific for the Ok^a antigen (Fig. 1).[21] MIMA-144 is an indirectly agglutinating Mab, while MIMA-25 and MIMA-149 are directly agglutinating Mabs. Their isotype, titer, score, and reactions with enzyme and chemically pretreated Ok(a+) RBCs are shown in Table 1. When tested in parallel with the original human anti-Ok^a , they were found to have different reactivity patterns when tested with enzyme or chemically modified RBCs. MIMA-25 was strongly reactive with Ok(a+) RBCs that were treated with trypsin, pronase, and neuraminidase, and weaker with papain, α_1-chymotrypsin, and 0.2 M DTT. MIMA-144 was weakly reactivity with Ok(a+) cells treated with papain, trypsin, and α_1-chymotrypsin. It was sensitive to treatment with pronase and neuraminidase, but resistant to treatment with 0.2M DTT. MIMA-149 was resistant to treatment with trypsin and pronase and weakly reactive with treatments of papain, α_1-chymotrypsin, neuraminidase, and 0.2M DTT. The reactivity of the polyclonal anti-Ok^a with Ok(a+) RBCs was unaffected by treatment with trypsin, α_1-chymotrypsin, pronase, and 0.2M DTT, weakened when treated with papain, and sensitive to treatment with neuraminidase.

By flow cytometry each antibody demonstrated a positive change in fluorescence when compared with the negative control (Fig. 2). MIMA-149 demonstrated the best intensity and specificity, with a sharp, narrow peak suggesting that its epitope is a short sequence of amino acids. MIMA-144 showed a broader pattern of reactivity indicative of an epitope of a wider amino acid sequence. MIMA-25 was much weaker, with only a slight right shift, probably due to the isotype and low titer of this antibody. Flow cytometry of the native HEK cells shows the presence of the Ok^a antigen on untransfected cells, and the expression seems to be stronger than the expression on normal human RBCs (Fig. 3).

None of the Mabs were reactive by immunoblotting (results not shown).

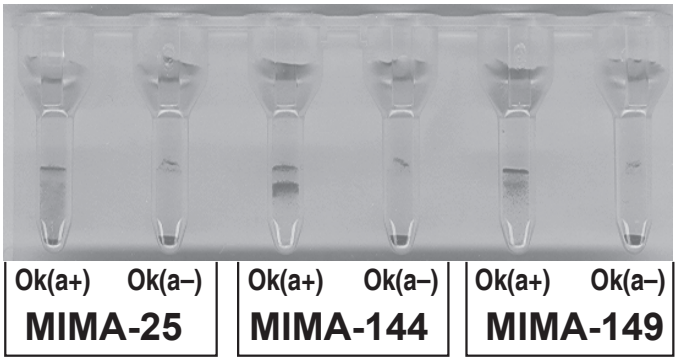

Fig. 1 Gel card photo of Mab reactivity with Ok(a+) and Ok(a–) RBCs.

Discussion

We had previously reported that the HEK cells, whether transfected with human cDNA or not, had a high enough expression of CD147 to initiate an immune response, and this is the reason why we were able to produce so many different Mabs to Ok^a. MIMA-25, -144, and -149 are new anti-Ok^a Mabs as demonstrated by their specific reaction with

Table 1. Isotype, titer (score), and effect of enzymes and chemicals on Ok(a+) RBCs

Antibody	Isotype	Titer (score)	Papain	Trypsin	α_1-Chymotrypsin	Pronase	Neuraminidase	0.2 M Dithiothreitol
MIMA-25	IgM	8 (29)	Weak	Resistant	Weak	Resistant	Resistant	Weak
MIMA-144	IgG2b	128 (77)	Weak	Weak	Weak	Sensitive	Sensitive	Resistant
MIMA-149	IgA	32 (55)	Weak	Resistant	Weak	Resistant	Weak	Weak
Human anti-Ok^a	Not tested	Not tested	Weak	Resistant	Resistant	Resistant	Sensitive	Resistant

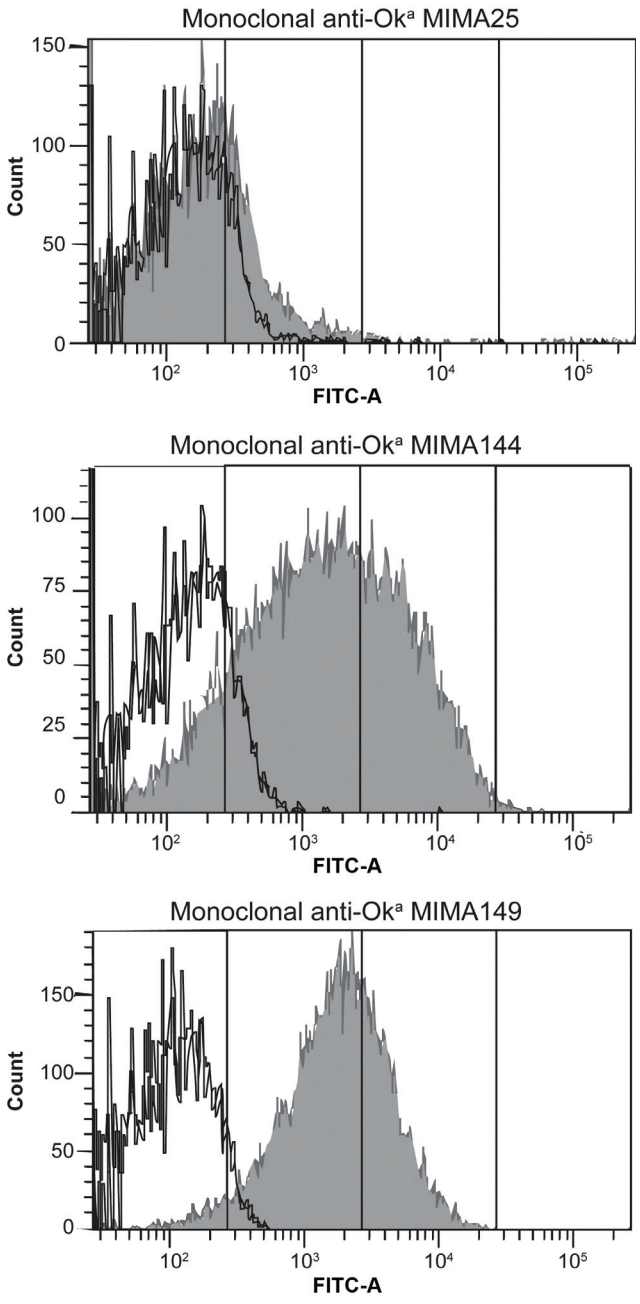

Fig. 2 Flow cytometry results with three Mabs against Ok(a+) and Ok(a–) RBCs. On the x-axis is the fluorescent intensity of the signal and on the y-axis is the number of cells counted. The shaded gray area represents the FITC-positive cell population. The unshaded graph represents the negative control cell population.

Ok(a+) RBCs, and their nonreactivity with six different Ok(a–) RBC samples (M. Uchikawa, personal communication). The reactivity pattern of each antibody is unique in the way it is affected when RBCs are modified by 0.2M DTT or various enzymes. All three antibodies react well in test tubes and gel cards. With our flow cytometry data, it is not surprising that MIMA-25 was much weaker, as it is an IgM Mab requiring very careful dilution controls for demonstration of the reactivity by flow cytometry. Serologically this antibody works best by hemagglutination in the presence of Lo-Ion to enhance the reactions.

None of these Mabs were reactive by Western blotting, suggesting that the epitope(s) they recognize may be conformation dependent.

Conclusions

The three Mabs are unique antibodies that will be useful for testing patients and screening blood donors to find Ok(a–) blood. MIMA-144 is an indirect agglutinating antibody, and MIMA-25 and MIMA-149 are directly agglutinating antibodies, and as such, are ideal reagents for rapidly typing RBCs.

Acknowledgments

We thank Robert Ratner for the preparation of the manuscript, tables, and figures. We thank Wu He for his assistance in performing the flow cytometry experiments, and Makoto Uchikawa for additional testing with Ok(a) RBC samples. We thank Karina Yazdanbakhsh for her review of and comments on the manuscript. This work was funded in part by a grant from the MetLife Foundation.

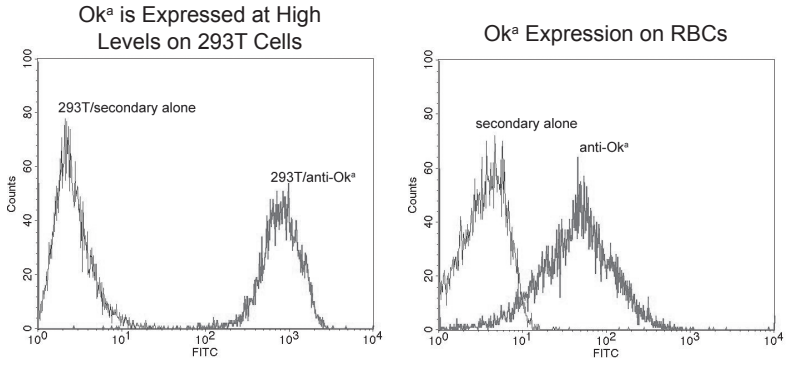

Fig. 3 Flow cytometry of transfected 293T cells and human Ok(a+) RBCs.

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